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Original Article

Prevention and treatment of experimental crescentic glomerulonephritis by blocking tumour necrosis factor-α

Ayman M. Karkar, Jennifer Smith and Charles D. Pusey

Renal Section, Division of Medicine, Imperial College School of Medicine, Hammersmith Hospital, London, UK

Abstract

Background. The mechanisms controlling progression of glomerulonephritis are poorly understood, but there is increasing evidence that tumour necrosis factor-α (TNF-α) plays a central role in many aspects of glomerular inflammation and scarring. We investigated the role of TNF-α in an experimental model of crescentic glomerulonephritis in Wistar Kyoto (WKY) rats by continuously blocking endogenous TNF-α, using its soluble receptor sTNFr p55, both before and after establishment of nephritis.

Methods. Glomerulonephritis was induced by a single intravenous injection of 0.1 ml nephrotoxic serum. In the first experiment, rats were pre-treated with sTNFr p55 2 mg/kg intraperitoneally 1 hour before induction of nephritis and on a daily basis thereafter until day 4. In the second experiment, a similar protocol was followed, but treatment with sTNFr p55 was continued until day 10. In the third experiment, treatment with sTNFr p55 was delayed until 4 days after induction of nephritis and continued until day 10. The effects of treatment on renal function, renal histology, cellular infiltration, activation and proliferation, and IL-1β expression were assessed by standard methods.

Results. In the first experiment, short-term treatment with sTNFr p55 caused a marked reduction in albuminuria and fibrinoid necrosis. It also reduced glomerular cell infiltration, activation and proliferation. In the second experiment, prolonged treatment with sTNFr p55 caused a sustained reduction in albuminuria and all histological and cellular parameters of glomerular inflammation; in particular it completely prevented the development of crescents. In the third experiment, delayed therapy of established nephritis with sTNFr p55 significantly reduced albuminuria and glomerular inflammation, including the prevalence of crescent formation. In both long-term experiments, there was less glomerular expression of IL-1β and lower serum concentrations of IL-β in sTNFr p55-treated rats.

Conclusions. This study shows that neutralization of endogenous TNF-α is effective in preventing acute glomerular inflammation and crescent formation, and in treating established disease, in a rat model of crescentic nephritis. These results may have therapeutic implications for human glomerulonephritis.

Keywords: crescentic glomerulonephritis; glomerular basement membrane; nephrotoxic serum; tumour necrosis factor-α; Wistar Kyoto (WKY) rat

Introduction

Rapidly progressive or 'crescentic' glomerulonephritis is characterized clinically by rapid deterioration of renal function, and histologically by mononuclear cell infiltration of the glomeruli, glomerular cell proliferation, crescent formation and eventually glomerulosclerosis. Crescentic nephritis occurs in a variety of human autoimmune disorders, including anti-glomerular basement membrane (GBM) disease, systemic vasculitis and systemic lupus erythematosus [1]. The mechanisms controlling progression of glomerulonephritis are poorly understood, but there is increasing evidence that cytokines and other growth factors, such as tumour necrosis factor-α (TNF-α), play a central role in modulating endothelial function, cellular infiltration and proliferation, and extracellular matrix production [2].

Tumour necrosis factor-α is a potent pro-inflammatory cytokine which is produced by many cell types including monocytes/macrophages, and renal mesangial and epithelial cells. It induces the expression of major histocompatibility complex (MHC) class I and II molecules, endothelial adhesion molecules and procoagulant activity of endothelium. TNF-α stimulates the release of other pro-inflammatory

Correspondence and offprint requests to: Professor Charles Pusey, Renal Section, Division of Medicine, Imperial College School of Medicine, Hammersmith Hospital, London W12 0NN, UK.

cytokines, chemokines and growth factors, including interleukin-1β (IL-1β), monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor-β (TGF-β) [3,4]. The biological effects of TNF-α are mediated by binding to specific receptors which are widely distributed. TNF-α binds to two types of receptor: TNF receptor type 1 and TNF receptor type 2, which have molecular weights of 55 kDa (p55) and 75 kDa (p75), respectively. Both receptors are necessary and act synergistically for cell proliferation and maturation, cytotoxicity and antiviral activity, but p55 is responsible for activation of NFκB and mediation of apoptosis [5]. Truncated fragments of the extracellular domains of TNF-α receptors are released as soluble forms, known as sTNFr p55 and sTNFr p75. Increased concentrations of both soluble receptors are detectable in serum and other biological fluids taken from patients with acute bacterial sepsis and chronic inflammatory diseases, such as rheumatoid arthritis and systemic lupus erythematosus [4]. *In vivo* administration of soluble TNF receptors proved to be of therapeutic benefit in an experimental model of sepsis [3]. Thus, these soluble inhibitors are believed to neutralize and clear excess TNF-α, and contribute to the control of the inflammatory response to injury.

There is a mounting evidence to implicate TNF-α in the pathogenesis of glomerulonephritis: (i) TNF-α is expressed in glomeruli of rodents with experimental nephritis, and is found in renal biopsies, sera and urine of patients with different types of glomerulonephritis [6–9]; (ii) *in vitro* and *in vivo* studies document that TNF-α is produced locally within inflamed glomeruli by mesangial and epithelial cells, as well as by infiltrating monocytes/macrophages [7,9]; (iii) systemic administration of TNF-α results in glomerular damage in rabbits [10] and exacerbates the degree of glomerular injury in nephrotoxic nephritis in rats [11]; and (iv) blocking endogenous TNF-α in nephrotoxic nephritis in rats ameliorates acute glomerular inflammation [12], and down-regulates glomerular IL-1β mRNA and circulating TNF-α concentrations [13].

Recent experimental studies have documented the effectiveness of inhibition of TNF-α in several different models of autoimmune disease [4]. These include collagen-induced arthritis, experimental allergic encephalomyelitis, experimental autoimmune uveitis and diabetes mellitus in NOD mice. Moreover, clinical trials in rheumatoid arthritis [14] and chronic inflammatory bowel disease [15] have shown that neutralization of endogenous TNF-α can control progression of disease, and reduces the production of other pro-inflammatory cytokines. Thus, blockade of TNF-α is a promising approach to the treatment of severe glomerulonephritis in man.

Two previous studies have examined the role of TNF-α in experimental crescentic glomerulonephritis. Lan *et al.* [16] used a model of accelerated nephrotoxic nephritis in Sprague–Dawley rats, in which there was a low (around 17%) proportion of crescents by day 14. Soluble TNF-α receptor type I, administered from 2 h before induction of nephritis, prevented crescent formation and also inhibited macrophage migration inhibitory factor (MIF) production. Le Hir *et al.* [17] used a model of accelerated nephrotoxic nephritis in mice that developed a low proportion (13%) of crescents. Animals deficient in TNF (TNF knockout) showed a significantly less severe glomerulonephritis than wild-type mice. However, there have been no studies of anti-TNF therapy in a model of severe crescentic glomerulonephritis and, in particular, no studies of delayed therapy in established disease.

Crescentic nephrotoxic nephritis (NTN) in Wistar Kyoto (WKY) rats closely resembles human anti-glomerular basement membrane (GBM) disease. This model was initially described by Kawasaki *et al.* in 1992 [18], and a detailed study of its natural history has recently been reported from our laboratory [19]. Crescentic nephritis is induced by a single intravenous injection of a small dose of anti-GBM antibody, which results in an influx of CD8+ cells and monocytes/macrophages into the glomeruli by day 1, which peaks at day 4. Severe necrotising glomerulonephritis develops by day 6, with crescent formation in >60% of the glomeruli within 2 weeks and renal failure by 4 weeks. Our previous studies, in a milder form of NTN in Sprague–Dawley rats, demonstrated the effectiveness of blocking TNF-α on acute glomerular inflammation [12]. Our aim in this study was to investigate the preventive and therapeutic benefits of continuous neutralization of endogenous TNF-α, using its soluble receptor p55, in a model of severe and progressive nephritis. Our results clearly show, for the first time, that continuous inhibition of TNF-α is effective in preventing crescent formation, even once nephritis is established.

Subjects and methods

Animals

WKY rats (Charles River, UK) weighing 180–200 g were used in all experiments. They were housed individually in metabolism cages for 24-h urine collections with free access to food and water. Blood samples were taken by tail vessel puncture under light isoflurane anaesthesia.

Reagents

Rabbit serum containing high titres of antibody to rat GBM (nephrotoxic serum, NTS) was prepared as described previously [20]. NTS was purified with special care to avoid contamination with endotoxin. It was tested for contamination with LPS by *Limulus Amoebocyte Lysate* assay, and the final concentration was always <5 pg of LPS per ml of NTS. The soluble TNF receptor p55 (sTNFr p55; a kind gift from Dr B. Scallon, Centocor, Inc., PA, USA) was produced by fusion of the extracellular domain of human p55 TNF receptor to the J sequence and constant domains of human IgG1. This fusion protein has a high affinity and neutralizing capacity towards human and rat TNF-α and TNF-β [21], and was free from contamination with endotoxin.

Treatment protocols

In these studies, sTNFr p55 was administered daily by intraperitoneal injection at a dose of 2 mg/kg, which has previously been shown to be effective in neutralizing endogenous TNF-α in rats [21]. The control rats received instead the vehicle, pyrogen-free phosphate-buffered saline (PBS). In pilot experiments, normal human IgG was used as the control injection, but did not show any differences from PBS. In the short-term prevention study, sTNFr p55 2 mg/kg i.p. was administered 1 h before induction of nephritis and daily thereafter until day 4, when the animals were killed. In the longer term prevention study, a similar protocol was used, but sTNFr p55 was administered daily until day 10. In the treatment study, sTNFr p55 was commenced on day 4 after induction of nephritis and continued on a daily basis until day 10.

Induction and assessment of glomerular injury

Glomerulonephritis was induced in WKY rats by a single intravenous injection of 0.1 ml of rabbit anti-rat GBM antibody (nephrotoxic serum). Blood and urine were collected at different time points, including days 4, 7 and 10, and animals were killed at either day 4 or 10. Glomerular injury was assessed functionally by measurement of albuminuria and serum creatinine, and by studying renal morphology by light microscopy. Albuminuria was measured by rocket immunoelectrophoresis using locally prepared polyclonal rabbit anti-rat serum albumin antiserum. Serum creatinine was measured using a standard colorimetric assay (Beckman autoanalyser).

Histology and immunohistochemistry

Kidney tissues were taken from experimental and control groups of rats at the time of killing. Tissue was fixed in 10% buffered formalin, embedded in paraffin wax, and 3-μm sections cut for light microscopic studies. Sections were stained with haematoxylin and eosin, and periodic acid Schiff for general morphology and for the incidence of glomerular fibrinoid necrosis and crescents, using standard techniques [12]. Immunoperoxidase staining was performed for the detection of glomerular cellular infiltration, using mAb ED1 for monocytes/macrophages and mAb OX8 for CD8+ lymphocytes (Serotec, Oxford, UK) [22]. The state of macrophage activation was examined using mAb ED3 to rat sialoadhesin (Serotec), and mAb to inducible nitric oxide synthase (iNOS) (Seralab, Crawley Down, UK). Glomerular cell proliferation was studied using mAb to rat proliferating cell nuclear antigen (PCNA) (Serotec).

The immunoperoxidase technique was performed as described previously [22,23]. Briefly, 4-μm-thick frozen sections were prepared from rat kidneys, mounted on poly-L-lysine-coated glass slides, air dried for 1 h, and then fixed for 10 min in cold acetone. The slides were rehydrated in PBS, treated for 30 min with hydrogen peroxide in PBS to block endogenous peroxidase activity, and washed again with PBS. Next, they were blocked with 20% normal sheep serum for 30 min and then exposed to different dilutions of ED1 (1:100), OX8 (1:50), ED3 (1:50), iNOS (1:50) and PCNA (1:50) or similar dilutions of control mAb. After overnight incubation in a humid chamber at 4°C, the slides were rinsed and washed three times with PBS, overlaid with peroxidase-conjugated polyclonal sheep anti-mouse antibody at 1:100 (Amersham International, UK) and incubated for 1 h, followed by three additional rinses and washes with PBS. At this point, sections were overlaid with substrate DAB (3,3'-diaminobenzidine tetrahydrochloride dihydrate, 97%) (Aldrich Chemical Co., Gillingham, UK) with hydrogen peroxide in PBS and incubated for 15 min at room temperature to allow colour development. Harris's haematoxylin was used as a counterstain. Glomerular cell infiltration, and the number of activated macrophages and proliferating cells were counted per 50 consecutive glomeruli in section.

Measurement of local and systemic synthesis of IL-1β

Glomerular synthesis of IL-1β was studied by immunohistology, using specific antibodies to rat IL-1β (Serotec). Glomerular expression of IL-1β was scored blind in a semi-quantitative scale from 0 to $+++$, depending on the extent and intensity of glomerular staining. Blood and urine samples were collected in endotoxin-free tubes, at different time points, for measurement of IL-1β concentrations (pg/ml) using a commercially available ELISA kit specific for rat IL-1β (Biosource, UK).

Statistical analysis

The data are presented as means together with standard errors (SE). The probability that differences between the groups were significant was calculated using the Wilcoxon Rank-Sum test.

Results

None of the treatments, either alone or in combination, caused any apparent disturbance to the health of the animals. Normal control rats, and rats injected with sTNFr p55 without NTS, showed no functional or morphological changes in the kidneys.

Effect of blocking TNF-α on acute glomerular inflammation

Glomerulonephritis was induced in 12 rats. One hour before induction of nephritis, one group ($n=6$) was pretreated with an i.p. injection of 2 mg/kg sTNFr p55, which was continued on a daily basis, whereas a control group ($n=6$) was given vehicle. All rats were killed 4 days after induction of nephritis. Blocking endogenous TNF-α caused a marked reduction in severity of nephritis. This was reflected by a significant reduction in urinary albumin excretion (22 ± 3 *vs* 69 ± 14 mg/day, $P<0.001$), and the prevalence of fibrinoid necrosis (2 ± 1 *vs* $30\pm5\%$, $P<0.001$). This was accompanied by significant reduction, per 50 glomeruli in section, in the number of cells within inflamed glomeruli (ED1+cells 356 ± 24 *vs* 538 ± 45, $P<0.001$; OX8+cells 19 ± 2 *vs* 49 ± 4, $P<0.001$), the state of cell activation (ED3+ cells 60 ± 2 *vs* 142 ± 8, $P<0.001$; iNOS+ cells 38 ± 4 *vs* 98 ± 4, $P<0.001$), and cell proliferation (PCNA+cells 114 ± 9 *vs* 282 ± 15, $P<0.001$). Results are summarized in Table 1.

Table 1. Effects of sTNFr p55 in prevention and treatment of crescentic nephritis in WKY rats

Treatment	Time of killing	*n*	Albuminuria (mg/24 h)			Fibrinoid necrosis (%)	Crescents (%)	Cells per 50 glomeruli in section				
			Day 4	Day 7	Day 10			ED1	OX8	ED3	iNOS	PCNA
Experiment 1												
sTNFr p55	day 4	6	22±3*	–	–	2±1*	–	356±24*	19±2*	60±2*	38±4*	114±9*
Control	day 4	6	69±14	–	–	30±5	–	538±45	49±4	142±8	98±4	282±15
Experiment 2												
sTNFr p55	day 10	5	2±1*	16±5*	16±7*	6±3*	0±0*	79±13*	27±2*	36±5*	11±3*†	45±5*
Control	day 10	5	35±15	160±7	149±6	44±7	41±5	205±18	39±1	112±6	27±6	144±41
Experiment 3												
sTNFr p55	day 10	6	21±8	86±33*	43±21*	3±1*	16±6*	94±28*	19±6*	25±6*	12±4*	125±9*
Control	day 10	6	20±5	259±14	246±17	38±3	82±2	355±18	36±2	108±5	43±4	286±16

*$P < 0.001$.

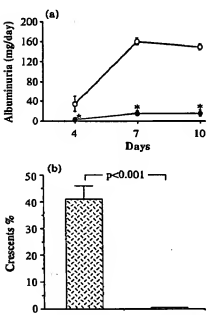

Fig. 1. Preventive effect of sTNFr p55 on urinary albumin excretion and crescent formation. (a) Significant reduction in albuminuria at days 4, 7 and 10. (b) Complete protection against crescent formation. Control group (○) and sTNFr p55 treated group (●). *$P < 0.001$.

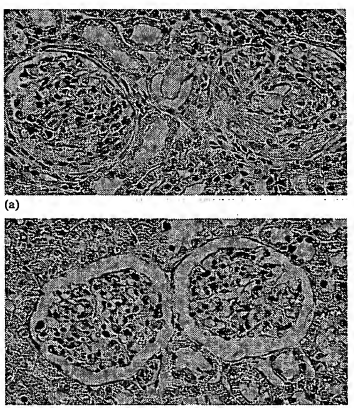

Fig. 2. Kidney sections from rats at day 10 in the prevention study. (a) Severe crescent formation and fibrinoid necrosis in the untreated control group. (b) Significant reduction in the prevalence of fibrinoid necrosis and complete prevention of crescent formation in the treated group (haematoxylin and eosin staining, ×250).

Effect of blocking TNF-α on progression of glomerulonephritis

A similar protocol to the previous experiment was used, with five rats in each group, but treatment with sTNFr p55 (or vehicle) was continued daily until day 10, when all rats were killed for assessment of glomerular injury. Continuous blocking of TNF-α caused a marked reduction in the development of albuminuria at day 4 (2±1 *vs* 35±15 mg/day, $P < 0.001$), day 7 (16±5 *vs* 160±7, $P < 0.001$) and day 10 (16±7 *vs* 149±6, $P < 0.001$) as shown in Figure 1a. Serum creatinine concentrations at day 10 were less in the sTNFr p55 treated group (30±2 *vs* 41±3 μg/ml, $P > 0.05$). There was a significant reduction in the prevalence of fibrinoid necrosis (6±3 *vs* 44±7%, $P < 0.001$). Strikingly, continuous inhibition of TNF-α completely prevented the development of crescents per 100 glomeruli in section (0±0 *vs* 41±5%, $P < 0.001$, Figure 1b). The effects on fibrinoid necrosis and crescent formation are illustrated in Figure 2. There was also a significant reduction, per 50 glomeruli in section, in the degree of

cellular infiltration (EDI+ cells 79±13 *vs* 205±18, $P<0.001$; OX8+ cells 27±2 *vs* 39±1, $P<0.001$), cellular activation (ED3+ cells 36±5 *vs* 112±6, $P<0.001$; iNOS+ cells 11±3 *vs* 27±6, $P<0.05$) and cellular proliferation (PCNA+ cells 45±5 *vs* 144±14, $P<0.001$). Results are summarized in Table 1.

Effect of blocking TNF-α in established glomerulonephritis

Four days after induction of nephritis, once disease was established, rats were divided into two groups. One group ($n=6$) was treated with an i.p. injection of sTNFr p55 on a daily basis, whereas the other group ($n=6$) received vehicle. All rats were killed on day 10. At day 4, albuminuria was similar in both groups (21±8 *vs* 20±5 mg/day, respectively). Continuous administration of sTNFr p55 from day 4 onwards caused a significant reduction in urinary albumin excretion at day 7 (86±33 *vs* 259±14 mg/day, $P<0.001$), and a further reduction at day 10 (43±21 *vs* 246±17, $P<0.001$), as shown in Figure 3a. Serum creatinine concentrations at day 10 were less in the sTNFr p55 treated group (65±4 vs 90±7 μg/ml, $P>0.05$). Neutralization of endogenous TNF-α caused a marked reduction in the prevalence of fibrinoid necrosis (3±1 *vs* 38±3%, $P<0.001$) and largely prevented the development of crescents per 100 glomeruli in section (16±6 *vs* 82±2%, $P<0.001$, Figure 3b). The histology is illustrated in Figure 4. There was also a significant reduction, per 50 glomeruli in section, in the degree of cellular infiltration (ED1+ cells 94±28 *vs* 355±18, $P<0.001$; OX8+ cells 19±6 *vs* 36±2, $P<0.001$), cellular activation (ED3+ cells 25±6 *vs* 108±5, $P<0.001$; iNOS+ cells 12±4 *vs* 43±4, $P<0.001$) and cellular proliferation (PCNA+ cells 125±9 *vs* 286±16, $P<0.001$). Results are summarized in Table 1.

Effect of blocking TNF-α on glomerular and systemic IL-1β synthesis

In the short-term study, in which rats were killed at day 4, immunohistological staining showed that IL-1β was minimally expressed within inflamed glomeruli, and there was no difference between the treated and control groups. Likewise, serum concentrations of IL-1β were barely detectable at this time point in both treated and control groups. In the long term prevention study, glomerular expression of IL-1β was increased at day 10, and there was a marked reduction in its expression in the sTNFr p55-treated group when compared with the control group (+ *vs* +++). Serum concentrations of IL-1β were elevated at day 10, and these were significantly less in the treated group when compared with the control group (32±3 *vs* 99±33 pg/ml, $P<0.02$). In the therapeutic study, there was also less glomerular expression of IL-1β in the sTNFr p55 treated group (+ *vs* +++). Similarly,

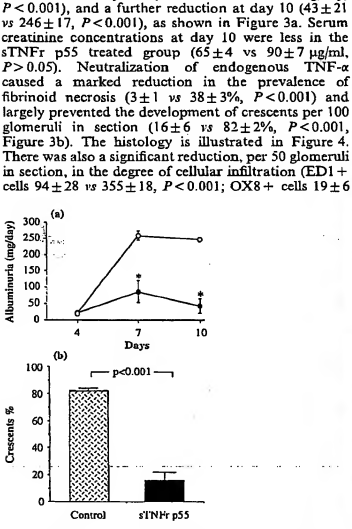

Fig. 3. Therapeutic effect of sTNFr p55 on urinary albumin excretion and crescent formation. (a) Significant reduction in albuminuria at days 7 and 10 (both groups had a similar degree of albuminuria at day 4 before starting treatment). (b) Marked protection against crescent formation. Control group (○) and sTNFr p55 treated group (●). *$P<0.001$.

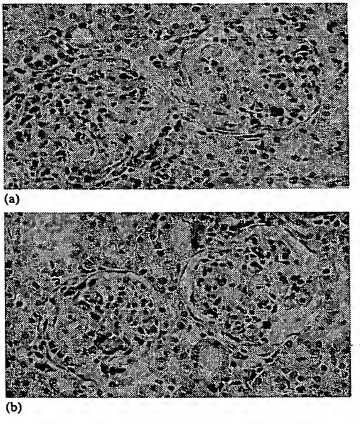

Fig. 4. Kidney sections from rats at day 10 in the treatment study. (a) Severe crescent formation and fibrinoid necrosis in the untreated control group. (b) Significant reduction in the prevalence of fibrinoid necrosis and crescent formation in the delayed treatment group (haematoxylin and eosin staining, ×250).

the treated group had lower serum concentrations of IL-1β, although these were not statistically different from the control group (44 ± 5 *vs* 82 ± 24, $P > 0.05$).

Discussion

Results from this study show that TNF-α is important both in acute glomerular inflammation, and also in the development of crescents, in an experimental model of severe crescentic glomerulonephritis. Our data show that pre-treatment with a neutralizing TNF receptor completely prevented crescent formation, and more importantly that continuous blocking of TNF-α once disease was established resulted in a significant reduction in all parameters of glomerular inflammation, including crescent formation. This is, to our knowledge, a novel finding and suggests new therapeutic approaches to the management of patients with rapidly progressive glomerulonephritis.

There is considerable evidence that TNF-α is largely responsible, together with IL-1β, for most acute inflammatory reactions. However, recent studies in experimental models of arthritis, encephalomyelitis and diabetes mellitus have shown that TNF-α is also involved in the chronic phases of these autoimmune diseases [4]. Furthermore, clinical studies in patients with rheumatoid arthritis and Crohn's disease have shown that blocking TNF-α, by monoclonal antibodies [14,15] or by its soluble receptor p75 [24], has beneficial effects. Thus, inhibition of TNF-α appears to be able to modulate autoimmune diseases, even when they are established. These effects may be mediated directly through TNF-α and/or through its capacity to stimulate the synthesis and release of other powerful mediators.

Tumour necrosis factor-α is capable of inducing the synthesis of many cytokines and chemokines, including TNF-α itself, IL-1β, TGF-β, MIP-2 and MCP-1 [3]. We have previously shown that sTNFr p55 is capable of completely neutralizing systemic TNF-α, down-regulating glomerular expression of IL-1β mRNA, and increasing glomerular expression of IL-1ra [13]. In the present study, we found that sTNFr p55 treatment was associated with a reduction in glomerular and systemic production of IL1β. Therefore, the beneficial effects of blocking TNF-α in this study could have been mediated, at least in part, by a reduction in IL-1β. Blocking TNF-α may also have reduced expression of certain chemokines, such as MCP-1 and MIP-2, and therefore reduced cellular infiltration. We have previously demonstrated a relationship between cellular infiltration and chemokine expression, and found that blocking TNF-α by sTNFr p55 reduced glomerular expression of the relevant chemokines [25]. In the present study, animals treated with sTNFr p55 showed less glomerular infiltration with CD8 cells and macrophages. In addition, the proportion of activated macrophages (as judged by ED3 and iNOS staining) was less in the treated animals. This suggests that blockade of TNF may also act by preventing activation of macrophages, which are known to be important in the progression of glomerulonephritis. Whether the reduction in proliferating glomerular cells is directly due to TNF blockade, or is secondary to effects on glomerular inflammation, is not yet clear.

Two other important actions of TNF-α, of relevance to the development of glomerulonephritis, are the induction of expression of endothelial adhesion molecules and of tissue factor. Previous work has demonstrated the benefit of blocking either LFA-1:ICAM-1 [26] or VLA-4:VCAM-1 [27] interactions in crescentic nephritis in the WKY rat. Blocking tissue factor has also been shown to reduce glomerular fibrin deposition and crescent formation in a mouse model of nephrotoxic nephritis [28]. Although the expression of tissue factor was not examined in the present study, the marked reduction in glomerular fibrin deposition suggests that effects on tissue factor expression could provide a further explanation for the benefit of blocking TNF.

In conclusion, these results are in agreement with our hypothesis that TNF-α plays a major role in acute inflammation and progression of injury in glomerulonephritis, and that its inhibition may have therapeutic benefits. This work provides new insights into mechanisms of glomerular inflammation and crescent formation, and suggests that inhibition of TNF-α is a strong candidate to be tested in the therapy of human glomerulonephritis.

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